Faculty of Resource Science and Technology

PRODUCTION AND TRIAL ENZYMATIC RETTING USING PECTINASE PREPARATION PRODUCED BY *ASPERGILLUS NIGER* UMAS FM1 USING SOLID SUBSTRATE FERMENTATION (SSF)

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Bachelor of Science with Honours

(Resource Biotechnology)

2010

Production and Trial Enzymatic Retting Using Pectinase Preparation Produced by *Aspergillus niger* UMAS FM1 Using Solid Substrate Fermentation (SSF)

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This project is submitted in partial fulfilment of the requirement of the degree of
Bachelor of Science with Honours
(Resource Biotechnology)

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Resource Biotechnology Programme

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Universiti Malaysia Sarawak

2010

DECLARATION

I hereby declare that this thesis is based on my original work except for quotations and citation, which have been duty acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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Acknowledgements

First of all, I would like to express my highest thanks and deepest appreciation to my supervisor, Dr Awang Ahmad Sallehin Awang Husaini for his excellent supervision and guidance throughout the entire duration of my project. I am very grateful that he has imparted me with many valuable knowledge and experiences in this project.

Apart from that, I would also like to express my sincere gratitude to the postgraduate students of the Molecular Genetics Laboratory, UNIMAS – Frazer and Nor Hidayah – for their valuable advice and generous help throughout the whole duration in completing my project. Not forgetting, the laboratory assistant, Ms. Shila for her generous assistance in the laboratory.

Last but not least, I would like to express my special gratitude to my parents for their moral support and encouragement since the commencing of this project. Besides, I would also like to extend my thankfulness to my coursemates for their generous support, care and concern during the course of the project.

List of Abbreviations

A.niger	*Aspergillus niger*
BSA	Bovine Serum Albumin
DNS	3,5-dinitrosalicylic acid
g	Gram
mg	Milligram
min	Minute
ml	Mililitre
µg	Microgram
µl	Microlitre
NaOH	Sodium hydroxide
nm	Nanometre
OD	Optical density
PDA	Potato Dextrose Agar
PG	Polygalacturonase
pH	Power of hydrogen ion
SMF	Submerged Liquid Fermentation
SSF	Solid Substrate Fermentation
˚C	Degree Celsius

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ABSTRACT

Pectinase is one of the main enzymes secreted during retting process. This enzyme is the one that responsible in attacking the pectin in the mesocarpic area of pepper skin and resulted in the decortication of pepper berries to produce the white pepper. This project is aimed to produce and optimize the pectinase produced by *Aspergillus niger* UMAS FM1 using solid substrate fermentation (SSF) for further application in the enzymatic retting in white pepper corn production. In this study, two parameters have been optimized. These are time of incubations and type of substrates used for solid substrate fermentation. Types of substrate that has been used are pepper waste, orange waste and pineapple waste. Pineapple waste and 4 days (96 hours) of time of incubation were found to be the best conditions to ease the pepper berries decortications. The pectinase was extracted and further analyzed using DNS assay and Bradford assay to determine the amout of reducing sugar and total protein respectively. Enzymatic activity has been calculated to determine the optimum production of pectinase. Trial enzymatic retting has been done using different concentrations of pectinase and different time of retting process. The optimum concentration of pectinase and days of retting were 0.110 µg/ml and in 6 days (144 hours) respectively.

Key words : Pectinase, *Aspergillus niger* UMAS FM1, Solid Substrate Fermentation (SSF), enzymatic retting

ABSTRAK

Enzim pectin ialah enzim utama yang dirembeskan semasa proses pereputan. Enzim ini berfungsi untuk menyerang pektin di dalam bahagian mesokarpik yang terdapat pada kulit lada dan ini akan menyebabkan berlakunya proses penanggalan kulit lada dan seterusnya menghasilkan lada putih. Projek ini bertujuan untuk menghasilkan dan mengoptimumkan enzim pektin yang dihasilkan oleh Aspergillus niger UMAS FM1 dengan menggunakan kaedah fermentasi pepejal subsrat untuk aplikasi proses pereputan enzim tersebut dalam penghasilan lada putih. Di dalam kajian ini, dua parameter telah dioptimumkan iaitu tempoh eraman dan jenis substrat yang digunakan untuk kaedah fermentasi pepejal substrat. Jenis-jenis substrat yang telah digunakan ialah sisa lada, sisa oren dan sisa nenas. Sisa nenas dan tempoh eraman selama 4 hari (96 jam) telah dikenalpasti sebagai keadaan yang terbaik untuk memudahkan proses penanggalan kulit lada. Enzim ini diekstrak dan dianalisa menggunakan ujian DNS dan ujian Bradford untuk menentukan jumlah gula penurun dan jumlah protein. Aktiviti enzim telah dihitung untuk menentukan penghasilan enzim pectin yang optimum. Proses ujikaji pereputan enzim dijalankan dengan menggunakan kepekatan enzim pektin yang berlainan dan tempoh proses pereputan yang berlainan. Kepekatan enzim pectin dan tempoh pereputan yang optimum ialah masing-masing 0.110 µg/ml dan dalam tempoh 6 hari (144 jam).

Kata kunci : Enzim pectin, Aspergillus niger UMAS FM1, fermentasi pepejal substrat, pereputan enzim

1.0 INTRODUCTION

Pepper is one of the world's most important spices and with India and Vietnam being the greatest pepper producing countries in the world and both pepper quality and process improvements in this region are highlighted. The pepper plant is a perennial woody vine growing to four meters in height on supporting trees, poles or trellises. It is a spreading vine, rooting readily where trailing stems touch the ground. The leaves are alternate, entire, five to ten centimeters long and three to six centimeters broad. The flowers are small, produced on pendulous spikes four to eight centimeters long at the leaf nodes, the spikes lengthening to seven to 15 centimeters as the fruit matures.

The white pepper obtained from pepper berries by removing the outer pericarp of the berries (Chithra *et al*., 2008). The pectinase is very important to ensure the enzymatic retting of pepper berries to produce white pepper corn can take place. Pectinase is the enzyme that helps the breakdown of pectins. On the other hand, pectins are structural carbohydrates which occur in the primary wall and middle lamellae of plant cells. Pectinase are of fungal origin from plant pathogen, *Aspergillus niger*. Based on the the paper by Kumpoun and Motomura (2001), the relationship of pathogenicity of fungus and cell degrading enzyme will assist the degradation of cell wall polysaccharides of pepper berries.

Solid-substrate fermentation is defined as those processes in which microbial growth and products formation occur on the surfaces of solid substrates in the near absence of free water. Due to this low amount of water available in solid-state bioprocessing, the classes of microorganisms that are most commonly used are fungi, bacteria and yeast (Martin *et al*., 2004). The pectinases can considerably reduce the white pepper processing and production time. This is because it will save mass amounts of water and energy and also improve the quality products. After the fresh pepper berries soaked into crude enzyme solution to enable pectinases to properly penetrate the skin, they will undergo decortications process. This process can be considered as milder treatment because it uses less energy and less water for flushing, making enzymatic retting a more sustainable alternative.

The main objective of this research project is to produce and optimize the pectinase produced by *Aspergillus niger* UMAS FM1 using solid substrate fermentation (SSF) for further application in the enzymatic retting in the white pepper corn production. There are several procedures that have been employed in this research project. They are preparation of media, preparation of *Aspergillus niger* UMAS FM1, solid substrate fermentation (SSF) which has two parameters, type of substrates media and time of incubations, production and enzymatic assay of pectinase and finally there is the trial enzymatic retting of pepper berries.

2.0 LITERATURE REVIEW

2.1 *Aspergillus niger*

Aspergillus niger is a filamentous ascomycete fungus that is easy to isolate from the environment. The genome size of *A. niger* is estimated to be between 35.5 and 38.5 megabases (Mb) divided among eight chromosomes or linkage groups that vary in size from 3.5 to 6.6 Mb (Baker, 2006). The rich amount of data resulting from these multiple *A. niger* genome sequences will be used for basic and applied research programs applicable to fermentation process development, morphology and pathogenicity. The physiological capabilities of filamentous fungi, which make them suitable for SSF, are growth at low water activity and low pH, the production of extra-cellular hydrolytic enzymes and sporulation.

Most filamentous fungi grow better at low water activities compared to bacteria and yeasts (Molitoris *et al*., 2000). For most filamentous fungi the optimal water activity for growth is between 0.93 and 0.98. The minimum water activity for growth ranges from 0.7 to 0.94. Most filamentous fungi grow reasonably well at low pH (Santos *et al*., 2008). This is advantageous since pH control is very difficult in SSF and the metabolism of microorganism often causes acidification (Manpreet *et al*., 2005).

Furthermore, the most well-known application of *A. niger* is as the major source of citric acid. Industrial citric acid production by *A. niger* represents one of the most efficient, highest yield bioprocesses in use currently by industry. This organism accounts for over 99% of global citric acid production which is more than 1.4 million tonnes per annum. *A. niger* citric acid production serves as a model fungal fermentation process. This is because it is commonly used for the production of native and foreign enzymes, including glucose oxidase and hen egg white lysozyme. Based on the previous study, the culture is rarely grown on a solid substrate, although this is still common practice in several countries, but is more often grown as a submerged culture in a bioreactor. In this way, the most important parameters can be strictly controlled and maximal productivity can be achieved. It also makes it far easier to separate the chemical or enzyme of importance from the medium and is therefore far more cost-effective.

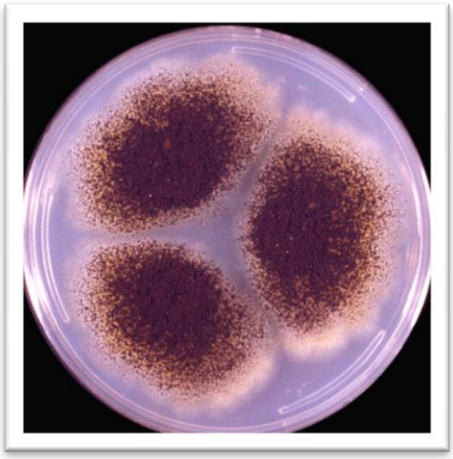

Figure 1 : *Aspergillus niger* on Czapek dox agar. Colonies consist of a compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads. Image retrieved from *www.mycology.adelaide.edu.au/gallery/photos/a...*

As a common member of the microbial communities found in soils, *A. niger* plays a significant role in the global carbon cycle. This organism is a soil saprobe with a wide array of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocellulose. A variety of these enzymes from *A. niger* are important in the biotechnology industry. *A. niger* is also an important model organism for several important research areas including the study of eukaryotic protein secretion in general, the effects of various environmental factors on suppressing or triggering the export of various biomass degrading enzymes, molecular mechanisms critical to fermentation process development, and mechanisms involved in the control of fungal morphology (Baker, 2006).

2.2 Pectinase

Pectinases are mixtures of enzymes that act on pectic substances, plant polysaccharides that maintain the integrity of the cell wall or middle lamellae. Pectic substances are acidic heteropolysaccharides of about 30,000 to 300,000 molecular weight and consist mainly of pectin, a polymer of D-galacturonic acid. Among fungal enzymes, pectinases are associated with development, fruit ripening and degradation of the pectic substances in the vegetal cell wall. This degradative process plays an important role in food technology, due to reduction in time of filtration and to the volume increase. This process leads to a more stable and concentrated product (Teixeira, 2001).

Furthermore, fungal pectic enzyme mixtures is used in commercial applications to remove pectic substances by typically serving as processing aids in fruit juice extraction and clarification (Milos & Mojovic, 2007). These pectinolytic enzymes are also used in the food industry, particularly for the isolation of essential oils and pigments from citrus. Fungi, bacteria and yeast are synthesizing and secrete pectinases. In addition to their industrial importance, pectinases are of interest because they participate in the infection process together with other plant cell wall-degrading enzymes, thus facilitating the installation of pathogens in plants (Barense, 2001). A common commercial source of pectic enzymes such as *Aspergillus niger* may contain activities many of which can play a role in degrading plant cell wall substances. These accompanying enzymatic activities material also can have a beneficial effect on the final characteristics of the food material after enzyme treatment (Ren *et al*., 2007).

Through studies with microorganisms, experimental assays demonstrated that the enzyme synthesis is correlated with the quality and concentration of the carbon and nitrogen sources. The studies of pectinases synthesis from *Aspergillus* show that it is induced mainly by pectin or pectin associated to other substances (Teixeira, 2001).

2.3 Solid Substrate Fermentation (SSF)

Solid Substrate Fermentation (SSF) is generally characterized by the growth of microorganisms on water insoluble substrates in the presence of varying amounts of free water. This type of fermentation is defined as those processes which utilize water insoluble materials for microbial growth in the absence of free water (Toca-Herrera *et al*., 2007). Thus, due to this low amount of water available in solid-state bioprocessing, the class of microorganisms that are most commonly used is fungi (Zheng & Shetty, 2000; Pandey *et al.*, 2001). Several agro-industrial waste and by-products such as orange bagasse (Martins *et al.*, 2002), sugar cane bagasse (Silva *et al.*, 2002) wheat bran (Cavalitto *et al.*, 1996) and other food processing waste (Zheng & Shetty, 2000) are effective substrates for depolymerizing enzyme production by solid-state fermentation.

SSF systems appear promising due to the natural potential and advantages they offer. The solid substrate acts as a source of carbon, nitrogen, minerals and growth factors, and has a capacity to absorb water that is necessary for microbial growth. As the microorganisms in SSF are growing under conditions similar to their natural habitats, they may be able to produce certain enzymes and metabolites more efficiently than in submerged fermentation. SSF has many advantages over SMF, including superior productivity, simple technique, low capital investment, low energy requirement and less water output, better product recovery and lack of foam build up and reported to be the most appropriate process for developing countries (Tanyildizi *et al.*, 2007).

A further advantage of SSF is that it is cheap and easily available, as substrates, such as agriculture and food industry by-products. Crude or partially purified enzymes produced by SSF have industrial applications. Such examples are pectinases used for fruit juice clarification and α-amylase is used for saccharification of starch. Inexpensive agriculture and agro-industrial residues represented one of the most energy-rich sources on the planet that can be used as a substrate in SSF. These residues are in fact, one of the best reservoirs of fixed carbon in nature.

In the SSF, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. The composition and concentration of media and fermentation conditions greatly affect the growth and production of extracellular enzymes from microorganisms. Cost and availability are important considerations, and therefore the selection of an appropriate solid substrate plays an important role in the development of efficient SSF processes. It is known that SSF is mainly confined to processes involving fungi and not suitable for bacterial cultures because of higher water activity requirements (Tanyildizi *et al.*, 2007).

Solid substrates usually consist of complex arrangements involving a number of macromolecules such as starch, cellulose, hemicelluloses, pectin, lignin, protein and lipid. These macromolecules or specific representatives amongst them usually provide the carbon and energy for microbial growth.

2.4 Enzymatic retting of pepper berries

Since many years, the production of white pepper of commerce is prepared either from freshly harvested berries or dried black pepper using special techniques such as retting, steaming and decortication. The recovery of white pepper from ripe berries is about 25%. Approximately 25 kg white pepper is obtained from 100 kg ripe berries. Steeping in water is the most popular and traditional technique that has been applied for preparing white pepper in which ripe pepper berries are soaked in water for 8 to 10 days and the outer skin is removed, washed and sun dried to reduce the moisture content to 10 to 12 % and to achieve a cream or white colour. The berries of Panniyur-1 are said to be ideal for the preparation of white pepper. The flavour is less pungent than that of black pepper.

White pepper consists of the seed only, with the skin of the pepper removed. This is usually accomplished by a process known as retting, where fully ripe peppers are soaked in enzyme for a particular period of time, during which the flesh of the pepper softens and decomposes. Rubbing then removes what remains of the fruit and the naked seed is dried. Alternative processes are used for removing the outer pepper from the seed, including decortication, the removal of the outer layer from black pepper from small peppers through mechanical, chemical or biological methods (Peter, 2001). This is an improved method with the modification from the traditional method.

The enzymatic retting of pepper depends on fermentation with pectinolytic microorganisms to remove pepper corn from the pepper berries (Schafer *et al*., 2007). Therefore, fungal pectic enzymes are added to the fermentation to remove the pulpy layer of the pepper that consists of pectic substances. Pectinase present in the enzyme preparation aids the degradation of pectin, the intercellular cementing substance present in the pulpy upper mesocarpic area of pepper skin (Kashyap *et al.*, 2000). Thus, pectinase is the main enzymes that attack the pectin that founds in cell wall of pepper and lead to the retting process of the pepper berries to produce the white pepper.

This retting process will be much easier and require short period of time if the conditions for production of pectinase has been successfully optimized. This is because the exact concentration of crude enzyme used for the retting process will assist pepper berries decortication. The overall aroma of such pepper was superior as compared to the aroma of white pepper produced according to the traditional procedure (Steinhaus & Schieberle, 2005).

3.0 MATERIALS AND METHODS

3.1 Preparation of media

In this experiment, Potato Dextrose Agar (PDA) media has been used to culture *Aspergillus niger* UMAS FM1. 12.9 grams of dehydrated Potato Dextrose powder have been suspended in 500 ml of distilled water. Then, the medium was boiled and stirred until the medium was completely dissolved. The pH medium was adjusted to pH 4.8. After that, sterilization process took place by autoclaving the medium at 121˚C for one and a half hour. The media was then poured into plates and stored at 4˚C in the refrigerator.

3.2 Preparation of *Aspergillus niger* UMAS FM1

The grown cultures of *Aspergillus niger* were taken from a Masters student, Nor Hidayah from the Molecular Genetic Laboratory for this experimental studies. The cultures were maintained on a 4.0% sterilized potato dextrose agar (PDA) medium at pH 4.8, based on works proposed by Ikram-ul-haq *et.al*. (2002). The media cultures of *Aspergillus niger* were incubated at 27ºC in the incubator for about a week before the cultures were subcultured into new plates.

3.3 Solid Substrate Fermentation (SSF)

3.3.1 Types of substrate media

Three types of substrate media were used in this project and they were pepper wastes, orange wastes and pineapple wastes. The pepper, orange and pineapple wastes were first washed and cleaned by distilled water. Then, the wastes were cut into small pieces to make them easier to be milled by the blending machine. The blended wastes were dried at 69˚C in an oven for 3 days (72 hours) until they were totally dried. The dried substrates were then milled and sieved to obtain mesh size and kept ready for the Solid Substrate Fermentation (SSF) process.

3.3.2 Time of incubations

The effect of incubation period on pectinase was determined by incubating the production medium for different incubation periods based on work done by Paranthaman *et al*. (2009) and the data are collected every 2 days of interval; 48 hours (day 2), 96 hours (day 4), 144 hours (day 6) and 192 hours (day 8).

3.4 Production and enzymatic assay of pectinase

Strains presenting large clearing zones were used for enzyme production assays on the solid substrate medium as described by Marcia *et al.* (1999). The cultivation under the solid substrate fermentation is carried out at 27°C for 8 days. At 2 days of intervals the solid fermented material, corresponding to one Erlenmeyer flask is mixed with 30 mL of distilled water (5g of fermented material/mL), stirred for 30 min, filtered using sterilized cloth and centrifuged at 4˚C for 20 min and using 6000rpm.

Polygalacturonase (PG) activity is determined by measuring the release of reducing groups from pectin using the 3,5-dinitrosalicyclic acid (DNS) reagent assay (Natalia *et.al.*, 2004). The reaction mixture containing 400 µl of 0.5 % citric pectin 67% methoxylated (Braspectina-Limeira/SP/Br) in 0.2M acetate buffer, pH 5.0 and 400 µl of crude enzyme solution, is incubated at 37ºC for 30 min (Soares *et al*., 1999). One unit of enzymatic activity (U) is defined as the amount of enzyme which released one m mol of galacturonic acid per minute. On the other hand, Pectin lyase (Pl) activity is determined by measuring the increase in absorbance at 575 nm of substrate solution (0.5 % citric pectin in 0.2M acetate buffer, pH 5.0) hydrolyzed by 400 µl enzyme solution, at 37°C. The enzyme production was expressed in units per gram of initial dry solid substrate (Ug^{-1}).

3.4.1 DNS assay

DNS assay was used to determine the amount of reducing sugars in crude enzyme (Solis-Pereyra *et al*., 2004). Firstly, the DNS reagent has to be prepared at room temperature. This reagent is the mixture of 2 g NaOH, 2 g DNSA, 0.4 g phenol and 0.1 g sodium sulphite in 200 ml of distilled water. The standard concentration of glucose was determined by using y-value of standard curve. 5 mg/ml of glucose has been used as glucose standard concentration. 1 ml of this solution has been taken out and put into a clean test tube. After that, 9 ml of distilled water were added into that solution. This solution was mixed thoroughly using vortex. Then, 5 ml of this solution was taken out and put into another test tube. 5 ml of distilled water were added into this test tube. After that, they were mixed by vortex.

The steps where 5 ml of glucose mixture added with 5 ml of distilled water were repeated for 4 times in different test tubes. Then, 800 µl of mixture from each test tube was added with 800 µl DNS reagent in a tightly capped test tube. The mixture was boiled at 100º C for 15 minutes to develop the red-brown color. 400 µl of 40% potassium sodium tartarate tetrahydrate (Rochelle salt) solution was added to stabilize the color. After cooling to room temperature, the absorbance was recorded with a spectrophotometer at 575 nm. These steps were used to determine the glucose concentration of crude enzyme produced by substituting the glucose solution with crude enzyme solution (Solis-Pereyra *et al*., 2004). The graph of standard glucose concentration was constructed (Appendix I).